Overproduction of N^{ϵ} -(Carboxymethyl)lysine-Induced Neovascularization in Cultured Choroidal Explant of Streptozotocin-Diabetic Rat

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Action of N^{ϵ} -(carboxymethyl)lysine (CML) adduct, an advanced glycation end product, was investigated on neovascularization of cultured choroidal explants in streptozotocin (STZ)-diabetic rat. The choroidal explants of early (4 weeks after an injection of 60 mg/kg STZ) and advanced (8 months after the STZ injection) diabetic rats, and age-matched normal rats were cultured in fibrin gel with Dulbecco's modified Eagle medium containing fetal bovine serum. The number of budded microvessel-like structures was counted and used as an index of *in vitro* neovascularization. Choroidal explants in the early diabetic stage released vascular endothelial growth factor (VEGF) and tended to increase tumor necrosis factor (TNF) α and platelet-derived growth factor (PDGF)-B, and concomitantly facilitated growth of sprout and buds, compared to the normal control. When choroidal explants were stimulated with CML-human serum albumin (HSA), its releasing effect was in the order VEGF>TNF α >PDGF-B. CML-HSA and CML-bovine serum albumin augmented the neovascularization in the cultured diabetic explant and their actions did not virtually differ. A monoclonal anti-CML antibody (6D12) inhibited the neovascularization in the advanced diabetes greater than that in the early diabetes. Inhibitory actions of anti-VEGF and anti-TNF α antibodies on the neovascularization were similar to that of the anti-CML antibody in the diabetes. In conclusion, CML adducts were accumulated and over-produced the actions of VEGF, TNF α and PDGF-B in the choroidal explant during diabetes in an age-dependent manner. TNF α and VEGF are likely to play a predominant role for the CML-induced choroidal neovascularization.

Key words choriocapillaris; neovascularization; N^{ϵ} -(carboxymethyl)lysine (CML); advanced glycation end product; diabetic retinopathy; streptozotocin

Neovascularization of retina has a causal role for visual impairment in proliferative diabetic retinopathy. Diabetic retinopathy is characterized by increased vascular permeability, thickening of the basement membrane, capillary occlusion, the formation of microaneurysm and neovascularization.¹⁾ The presence of persistently high blood level of glucose in patients with diabetes mellitus and animal models of diabetes has been implicated in the development of degenerative microvascular changes during diabetic complication including diabetic retinopathy.^{2–4)} One mechanism of tissue damage such as diabetic retinopathy, linking hyperglycemia may be the formation of advanced glycation end products (AGEs).⁵⁾ N^{ϵ} -(carboxymethyl)lysine (CML), one of major structures of AGEs, and other AGE structures are accumulated in patients with diabetes mellitus and animal models of diabetes, which may develop degenerative microvascular changes of retina^{2,3)} such as those in diabetic retinopathy^{4,6,7)} and could be implicated in the pathogenesis of diabetic retinopathy.^{8,9)} We have previously reproduced using *in vitro* tissue culture system that streptozotocin (STZ)-diabetic rat grows and develops retinal neovascularization in culture, suggesting participation of accumulated CML in the overproduction of retinal neovascularization.^{10,11)} If CML-modified proteins in the retinal tissue induce overproduction of neovascularization in the diabetes, it is important to investigate conditions of choroid during the period of diabetes. The neovascularization is, in deed, overproduced in cultured

choroidal explant of STZ-diabetic rat.¹²⁾ Administration of CML adduct facilitates to proliferate CD34⁺ cells such as endothelial progenitor cells, to form and develop immature microvessels in cultured choroidal explant of Wistar normal rat.¹³⁾ There are circumstantial evidences that vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF) α and platelet-derived growth factor (PDGF)-B facilitate the choroidal neovascularization in culture (our unpublished data),^{14,15)} that the glycated sera in the early stages of spontaneously diabetic GK and STZ-diabetic Wistar rats modify the activity of macrophages to release growth factors and facilitate formation of precapillaries from vascular endothelial cells,^{16–18)} and that AGEs induce the growth and precapillary formation of microvascular endothelial cells, although its extracted structure(s) was not identified.¹⁹⁾

In the present study, we investigated inhibitory effects of monoclonal anti-CML antibody (6D12) on the growth of sprouting and buds (neovascularization) of choroidal microvessels in culture in the early and advanced stages of STZ-diabetic rats to understand the roles of accumulated CML for the overproduction of choroidal neovascularization. Actions of CML on the release of VEGF, TNF α and PDGF-B from choroidal explants were also investigated to understand the mechanism of CML adducts for choroidal neovascularization during the period of diabetes.

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MATERIALS AND METHODS

Preparation of Hyperglycemic Rats A single dose of STZ (Sigma MO, U.S.A., 60 mg/kg body weight with saline) was injected intravenously into Wistar male rats (6 weeks of age; Kiwa Laboratory Animal Science Co., Wakayama). Early stage of STZ-diabetic Wistar rats (9–14 weeks of age; fed blood glucose level, 334–677 mg/dl), age-matched young normal Wistar rats (9–13 weeks of age; fed blood glucose level, 105–181 mg/dl), advanced stage of STZ-diabetic Wistar rats (9 months of age; fed blood glucose level, 418–577 mg/dl) and age-matched aged normal rat (9 months of age; fed blood glucose level, 106–130 mg/dl) were used in the present study. Blood glucose levels were measured in these animals with Beckman glucose analyzer (type II, Beckman Coulter, Tokyo, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Faculty of Pharmaceutical Sciences, Hokuriku University.

Preparation of Monoclonal Anti-AGE Antibody and CML-Modified Proteins The monoclonal anti-AGE antibody was prepared and characterized as reported previously.²⁰ Briefly, splenic lymphocytes from Balb/c mice immunized with AGE-bovine serum albumin (BSA) were fused to myeloma P3U1 cells. The hybrid cells were screened, and two cell lines that had a positive reaction to AGE-BSA, but a negative reaction to BSA were selected through successive subcloning. Each antibody was produced in ascitic fluid of Balb/c mice and further purified by protein A sepharose column chromatography. One of these antibodies designated as 6D12 was used in the present study. CML-human serum albumin (HSA) was prepared as described previously.^{13,21,22} Briefly, HSA (175 mg, fraction V, Sigma, St. Louis, MO, U.S.A.) was incubated at 37 °C for 24 h in 1 ml of 0.2 M sodium phosphate buffer (pH 7.8) with 0.15 M glyoxylic acid and 0.45 M NaCNBH₃ followed by dialysis against phosphate buffered saline (PBS). Amino acid analysis²⁰ indicated that CML contents of CML-HSA and CML-BSA prepared were 18.01 mole CML per mole HSA and 34.5 mole CML per mole BSA, respectively.

Preparation of Explants of Choroidal Capillaries Explants of choroidal capillary of deeply anesthetized STZ-diabetic and age-matched control normal Wistar rats were prepared as previously reported.^{12,13} Blood vessels, connective and fatty tissues in the outside of sclera in eye balls of these rats were removed in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) containing 10% heat-inactive fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, U.S.A.), 160 U/ml benzylpenicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo). After removal of the cornea, lens, corpus vitreum and retina from the eyeballs, explants of choroidal tissues were isolated with 10% FBS-DMEM in the presence of antibiotics under optical dissection microscope. Area of isolated choroidal explants was approximately 0.16 mm².

Tissue Culture of Explants of Choroidal Capillaries The similar size explants of choroidal capillaries were cultured as reported previously.^{12,13} They were plated on fibrin gels prepared by mixing 3 mg fibrinogen (0.3 ml, Sigma), and 1 U thrombin (Sigma) per ml DMEM containing antibiotics in 16-mm dish (Corning, Corning, NY, U.S.A.). The same

volumes of a mixture of the above concentrations of fibrinogen and thrombin solutions were overlaid and solidified. The choroidal explants were cultured with 1% FBS- or 5% FBS-DMEM (0.5 ml) containing antibiotics and 300 µg/ml ϵ -amino caproic acid at 37 °C under 5% CO₂ and 95% air. In some experiments, CML-HSA, CML-BSA, CML-free BSA anti-CML antibody, polyclonal goat anti-mouse VEGF neutralizing antibody (R & D system, Minneapolis, MN, U.S.A.), polyclonal rabbit anti-mouse TNF α antibody (Genzyme, Cambridge, MN, U.S.A.) or polyclonal rabbit anti-human PDGF-B antibody (Genzyme) were administered into the culture medium. The cultured medium was replaced every other day.

Assessment of Angiogenesis Number of vessel-like structures newly budded from cultured explants of choroidal capillary was counted with an Olympus camera equipped with a CKS microscope (Olympus, Tokyo) under $\times 40$ magnifications. The number of budded microvessel-like structures per explant was used as an index of *in vitro* choroidal neovascularization.^{12,13,15} Typical photographs of microvessel-like structures in cultured choroidal explants have been published previously.^{12,13} Cells composed in the microvessel-like structures are positive to antibody against CD 34,¹³ which is a marker of endothelial progenitor cells and vascular endothelial cells.²³ The cells have characteristics of fibroblast-like cells which are actively excreting background substances rather than mature endothelial cells. Electron microscope sections of the vessel-like structures in fibrin gel show additional evidences that these sprouted structures had narrow lumina occasionally surrounded by attenuated cellular extensions. These observations indicate that the vessel-like structures are immature microvessels consisted of CD34⁺ cells.¹³

Measurement of VEGF, TNF α and PDGF-B Contents in Media Cultured with Choroidal Explants by Enzyme-Linked Immunosorbent Assay Choroidal explants in early stage of STZ-diabetic and age-matched normal rats were cultured with 1% FBS- or 5% FBS-DMEM in the presence or absence of CML-HSA (1 µg/ml) for 6 d. Their media during 4 to 6 d in culture were harvested and stored at –70 °C. The concentrations of immunoreactive VEGF, TNF α and PDGF-B in the culture media were determined by colorimetric competitive enzyme-linked immunosorbent assays (ELISA) for mouse VEGF, rat TNF α and human PDGF-B (R&D system), respectively. ELISA for human PDGF-B recognizes also rat PDGF-B.²⁴

Statistical Analysis All values were expressed as means $\pm$ S.E.M.. Differences between group data were evaluated by one-way analysis of variance followed by the multiple range test of Scheffe at $p=0.05$ or 0.01 . A value of $p<0.05$ was considered statistically significant.

RESULTS

Actions of CML-Modified Protein on the Release of VEGF, TNF α and PDGF-B from Cultured Choroidal Explants in the Early Stage of Diabetic Rat ELISA directly measured contents of VEGF, TNF α and PDGF-B in the culture media of explants of the choroidal capillary derived from early diabetic rats and normal rats, during 4 to 6 d in culture. The content of VEGF in the culture media of

Fig. 1. Contents of VEGF, TNF α and PDGF-B Released from CML-HSA-Treated Choroidal Explants of Early-STZ-Diabetic and Age-Matched Normal Rats

Choroidal explants were cultured in fibrin gel with 1% FBS-DMEM in the presence or absence of CML-HSA (1 μ g/ml) (left) and with 5% FBS-DMEM (right) for 6 d. Contents of VEGF, TNF α and PDGF-B were measured in cultured media during days 4–6 in culture by ELISA kits. Values represent means $\pm$ S.E.M. of 8–13 (left) and 7–20 (right) data. * p <0.05, ** p <0.01: Significantly different from the value in the diabetic and normal choroids without CML-HSA. † p <0.05, †† p <0.01: Significantly different from the value in the normal choroid.

choroidal explants in the early diabetic rats were significantly greater than that in the normal control rats under conditions of 1% FBS- and 5% FBS-DMEM (Fig. 1). However, the contents of TNF α and PDGF-B in the media of cultured diabetic explants only tended to increase compared with those of the normal control under these FBS concentrations. The application of CML-HSA (1 μ g/ml) significantly increased the contents of VEGF, TNF α and PDGF-B released from the diabetic explant. The effects of CML-HSA on the release of VEGF and TNF- α were greater than that on the release of PDGF-B in the diabetic explants. The effects of CML-HSA on the release of these factors were greater than those in the normal explant (Fig. 1). These results indicate that the action of CML-HSA on the release of VEGF, TNF α and PDGF-B from choroidal explant was augmented in the early diabetic stage.

Actions of CML Adduct on Neovascularization of Cultured Choroidal Explants in the Early Stage of Diabetic Rat Choroidal explants of the early stage of STZ-diabetic and age-matched normal rats were cultured in DMEM with 1% FBS in the presence or absence of CML-HSA (0.1–1 μ g/ml) for 6 d. CML-HSA (18.01 mole CML per mole HSA; 0.1–0.3 μ g/ml) increased number of microvessels in the choroidal explant of early diabetic rat in a concentration-dependent manner on the 6th day in culture (Fig. 2). CML-HSA at 0.3 μ g/ml showed a maximal effect in the diabetic explant. In contrast, only a higher concentration of CML-HSA (1 μ g/ml) significantly increased the number of microvessels in the normal explant (Fig. 2). When the normal value without CML-HSA and the diabetic value with

Fig. 2. Concentration-Dependent Actions of CML-HSA on Number of Budded Microvessels of Cultured Choroidal Explants in Early-Diabetic and Age-Matched Normal Rats

Choroidal explants in the diabetic (●) and normal (○) rats were cultured in fibrin gel with 1% FBS-DMEM and microvessels in explants were counted on 6 d in culture. Values represent means $\pm$ S.E.M. of 6–26 data. C represents control without CML-HSA. * p <0.05, ** p <0.01: Significantly different from the value of the corresponding control without CML-HSA.

0.3 μ g/ml CML-HSA were estimated to 0% and 100% (Fig. 2), respectively, ED₅₀ value of CML-HSA with 95% confident limit was 0.075 μ g/ml (0.034–0.17 μ g/ml) for the diabetic explant and was significantly smaller than that for the normal explant [0.65 μ g/ml (0.26–1.60 μ g/ml)]. These results demonstrate that action of CML-HSA on the choroidal neovascularization was augmented in the early diabetic stage.

To clarify action of CML adducts in CML-modified proteins on the choroidal neovascularization, we compared the actions of different concentrations of CML adducts in CML-BSA and CML-HSA on the number of microvessels. CML-BSA (34.5 mole CML per mole BSA; 0.1–0.3 μ g/ml) increased the number of microvessels of the diabetic explant. The activity of CML-BSA (0.1 μ g/ml) with 34.5 mole CML per mole BSA was greater than that of CML-HSA (0.1 μ g/ml) with 18.01 mole CML per mole HSA (Fig. 3A). CML-free BSA (1 μ g/ml) did not affect the vessel number. The activities of CML-BSA, CML-HSA and CML-free BSA were plotted against the log concentrations of CML on the 4th day in culture (Fig. 3B). CML (0.027–0.154 μ M) increased the number of microvessels of diabetic explant in a concentration-dependent manner and the log concentration–neovascularization curve of CML in the diabetic explant was sifted to left side compared with that in the normal explant. The results indicated that the CML adducts in CML-modified proteins has a crucial role for the choroidal neovascularization *in vitro* in the diabetic rat.

Influence of Advanced-Diabetic Stage and Aging on Choroidal Neovascularization in Culture The onset times of growth of choroidal capillary in the early and advanced diabetic rats were similar to those in the age-matched normal rats, respectively (Fig. 4). The advanced stage of diabetic rats increased the number of microvessels in a time-dependent manner and the number in the advanced stage was significantly greater than those in the early stage and young normal control. However, the number of microvessels in the advanced diabetic stage tended to decrease but did not significantly differ from that in the age-matched normal aged rats

Fig. 3. Actions of CML-HSA and CML-BSA on the Number of Microvessels in Cultured Choroidal Explant of Early-Diabetic Rats (A), and Actions of CML Adducts on the Number of Microvessels in Cultured Choroidal Capillaries of the Diabetic and Age-Matched Normal Rats (B)

These explants were cultured in fibrin gel with 1% FBS-DMEM for 8 d with CML-BSA (0.1–0.3 $\mu\text{g/ml}$; ■), CML-HSA (0.1–0.3 $\mu\text{g/ml}$; ●) or CML-free BSA (1.0 $\mu\text{g/ml}$; □) or without them (○). Microvessels in explants were counted on 0, 2, 3, 4, 6 and 8 d in culture (A). Using data on 4 d in culture, microvessel numbers against log CML concentrations were plotted in the diabetic (CML-BSA, ■; CML-HSA, ●; CML-free BSA, □; without them, ○) and normal choroidal explants (CML-HSA, ▲; without it, △) (B). Values represent means $\pm$ S.E.M. of 13–28 (A) and 6–26 (B) data. C in Fig. 3B represents control without CML. * $p < 0.05$, ** $p < 0.01$: Significantly different from the value in the corresponding control with CML-free BSA.

Fig. 4. Neovascular Activity of Choroidal Explants in the Early Stage (A) and Advanced Stage (B) of STZ-Diabetic Rats

The choroidal explants of the early diabetic rat (A: ●), the age-matched young normal rat (A: ○), the advanced diabetic rat (B: ●) and the age-matched aged normal rat (B: ○) were cultured in fibrin gel with 5% FBS-DMEM for 6–8 d. The number of microvessels per explant was counted on days 2–8. The values were expressed as means $\pm$ S.E.M. of 10–15 data (A) and 8–12 data (B). * $p < 0.05$, ** $p < 0.01$: Significantly different from the normal value on the corresponding day in culture.

(Fig. 4B). These results indicate that the choroidal neovascularization is increased by both the diabetic stage and aging.

Inhibitory Effect of Anti-CML Antibody on the Neovascularization of Choroidal Explants in the Early and Advanced Diabetic Stages To investigate factors promoting neovascularization in the diabetic choroid, effects of anti-CML antibody on the neovascularization were investigated in the early and advanced diabetic stages. Anti-CML antibody inhibited CML-increased number of microvessels of cultured choroidal explant of normal rat.¹³⁾ The anti-CML antibody (3 $\mu\text{g/ml}$) also significantly inhibited the number of microvessels of cultured choroidal explant in the early diabetic rats to the level of the age-matched young control (Fig. 5A). The same concentration of anti-CML antibody did not affect

Fig. 5. Inhibitory Effects of Anti-CML Antibody on the Increased Number of Microvessels in the Early Stage (A) and the Advanced Stage (B) of Diabetic Rats

The choroidal explants were cultured in fibrin gel with 5% FBS-DMEM in the presence (●) or absence (○) of anti-CML antibody (3 $\mu\text{g/ml}$) for 6–8 d. The number of microvessels per explant was counted on days 2–8. The values were expressed as means $\pm$ S.E.M. of 12–30 data (A) and 10–21 data (B). * $p < 0.05$, ** $p < 0.01$: Significantly different from the value without the antibody on the corresponding day in culture.

the vessel number in the age-matched normal young rats (our unpublished data). The CML antibody also significantly decreased the number of microvessels of cultured explants in the advanced diabetic rats and the efficacy of inhibition was greater than that in the early diabetes (Fig. 5B). These results demonstrate that the accumulated CML adducts increased the neovascularization of cultured choroidal explants, indicating that the action of accumulated CML in the advanced diabetic choroid was greater than that in the early diabetic choroid.

Inhibitory Effects of Anti-VEGF and Anti-TNF α Antibodies on the Neovascularization of Choroidal Explant in the Diabetes We have previously reported that VEGF, TNF α and PDGF-B facilitate the choroidal neovasculariza-

Fig. 6. Effects of Anti-VEGF, Anti-TNF α and Anti-PDGF-B Antibodies on the Number of Microvessels in CML-HSA-Administered Choroidal Explants of Early Diabetic (A) and Age-Matched Normal Rats (B), and Choroidal Explants Without Administered CML-HSA in Early Diabetic Rats (C)

These explants were cultured in fibrin gel with 1% FBS-DMEM containing CML-HSA (1 μ g/ml) (A,B) or 5% FBS-DMEM (C) in the presence of anti-VEGF (2; 0.3 μ g/ml), anti-TNF α (3; 1:1000) and anti-PDGF-B antibodies (4; 3.3 μ g/ml) and absence of them (1; control) for 6 d. Microvessels in explants were counted on 6 d in culture. Values represent means $\pm$ S.E.M. of 13–23 (A), 24–34 (B) and 11–20 (C) data. * p <0.05, ** p <0.01: Significantly different from the value in the corresponding control without antibody (1).

tion in culture (our unpublished data).^{14,15} Since CML releases these angiogenic factors from cultured choroidal explant of the early diabetic rat (Fig. 1), their actions on CML-stimulated choroidal neovascularization in culture were compared between the early diabetic stage and the normal state, using their antibodies on the 6th day in culture. Anti-VEGF (0.3 μ g/ml), anti-TNF- α (1:1000) and anti-PDGF-B (3.3 μ g/ml) antibodies inhibited the CML-increased number of microvessels in the diabetic choroid (Fig. 6A). The inhibitory effect of anti-PDGF-B antibody had a tendency to be greater than those of anti-VEGF and anti-TNF α antibodies. At these concentrations, anti-VEGF and anti-TNF α antibodies inhibited the CML-increased number of microvessels of the young normal choroid with the similar inhibitory efficacy but the anti-PDGF-B antibody appeared to have a weaker potency (Fig. 6B). When CML-HSA was not applied to the cultured diabetic explant, anti-VEGF and anti-TNF α antibodies inhibited the number of microvessels but the anti-PDGF antibody did not significantly decrease it (Fig. 6C). Time-dependent actions of these antibodies were compared in the cultured diabetic explant without administration of CML-HSA. Anti-VEGF (0.3 μ g/ml) and anti-TNF α (1:1000) inhibited the number of microvessels in a time-dependent manner (Figs. 7A,B). However anti-PDGF-B (3.3 μ g/ml) significantly inhibited it on 3–4 d but not on 6–8 d in culture (Fig. 7C). These results demonstrated that the anti-VEGF and anti-TNF α antibodies inhibited proliferation of cells in microvessels but the anti-PDGF-B antibody delayed the onset time of sprouting microvessels. The rank order of inhibitory potency of these antibodies on the neovascularization in the early diabetic state was anti-VEGF $\geq$ anti-TNF α >anti-PDGF-B antibodies. The same concentrations of these antibodies did not affect the age-matched young normal neovascularization without CML (data not shown).

In the advanced diabetes, anti-VEGF (0.3 μ g/ml) and anti-TNF α (1:1000) antibodies inhibited choroidal neovascularization with a greater efficacy than they did in the early diabetes (Fig. 8). The inhibitory efficacy of anti-CML antibody

Fig. 7. Time-Dependent Effect of Anti-VEGF (A), Anti-TNF α (B) and Anti-PDGF-B (C) Antibodies on the Number of Microvessels in Choroidal Explants of Early Diabetic Rats

These explants were cultured in fibrin gel with 5% FBS-DMEM in the presence (●) or absence (○) of anti-VEGF (0.3 μ g/ml), anti-TNF α (1:1000) and anti-PDGF-B (3.3 μ g/ml) antibodies for 8 d. Values represent means $\pm$ S.E.M. of 7–11 (A), 12–17 (B) and 12–20 (C) data. * p <0.05, ** p <0.01: Significantly different from the value in the corresponding control without antibodies.

Fig. 8. Inhibitory Effects of Anti-VEGF and Anti-TNF α Antibodies on the Increased Number of Microvessels in the Advanced Stage of Diabetic Rats

Explants were cultured in fibrin gel with 5% FBS-DMEM in the presence of anti-VEGF (2; 0.3 μ g/ml) and anti-TNF α (3; 1:1000) and absence of them (1; control) for 6 d. Microvessels in explants were counted on 6 d in culture. Values represent means $\pm$ S.E.M. of 12–39 data. ** p <0.01: Significantly different from the value in the corresponding control without antibody (1).

was similar to those of anti-VEGF and anti-TNF α antibodies (Figs. 5, 8). Both antibodies did not affect the onset time of sprouting of microvessels in the advanced diabetic stage (data not shown). These results demonstrate that the actions of VEGF and TNF α were parallel with that of CML during the diabetic period, suggesting that tissue-accumulated CML initiates choroidal neovascularization through the release of TNF α , VEGF and PDGF-B from the choroidal explants in the diabetic rat.

DISCUSSION

The present study demonstrates that in the STZ-diabetic stage the action of CML adducts was augmented and promoted neovascularization in the cultured choroidal explant in an age-dependent manner. Several lines of evidence support this notion: (1) CML-HSA increased the release of angiogenic factors and the choroidal neovascularization in the diabetic rat. The action of CML-HSA was greater than that in the age-matched normal control rat (Figs. 1–3). (2) Actions of CML-HSA and CML-BSA did not virtually differ in their

growth properties, depending on the content of CML (Fig. 3). (3) Application of anti-CML antibody inhibited the CML-facilitated and diabetic stage-facilitated neovascularization (Fig 5).¹³⁾ (4) Non-immune mouse IgG and endotoxin potentially contaminating in the CML-modified proteins and the CML antibody did not influence the choroidal neovascularization (data not shown).

The initial study by Ikeda *et al.* demonstrated that 6D12 recognizes CML as one of its epitopes.²⁰⁾ However, the recent study has revealed that *N*^ε-(carboxyethyl)lysine (CEL) also serves as an epitope of this monoclonal antibody,²⁵⁾ indicating the common portion of CML and CEL being an epitope of 6D12. Since the anti-CML antibody effectively inhibited the CML-induced and diabetic stage-induced choroidal neovascularization, it is clear that CML is actively involved in these phenomena. It is not clear at the present time, however, whether CEL-modified proteins are involved in the choroidal neovascularization.

The present study shows that the actions of CML-HSA were augmented in the choroids obtained from the rats in the early diabetic stage. One of the mechanisms of the diabetic stage-enhanced activity may be associated with the action of tissue-accumulated CML adducts in the diabetic explant, being supported by the evidences that AGE and TNF α up-regulate messenger RNA and protein levels of receptor for AGE (RAGE).^{6,26)} Diabetic rats in the advanced diabetic stage maintain higher blood level of glucose (418—577 mg/dl) for 8 months. The activity of accumulated CML adducts in the advanced diabetic stage was greater than that in the early diabetic stage. The anti-CML antibody inhibited completely the choroidal neovascularization in culture with a greater efficacy in the advanced diabetes (Fig. 5). These results demonstrate that the amount of accumulated CML in the choroidal explant of the advanced diabetes is greater than that in the early diabetic stage. However, the choroidal activity in the advanced diabetic stage did not differ from that in the age-matched aged state, where the aged choroidal neovascularization in culture was also inhibited by the anti-CML antibody (our unpublished data). The similar results have been reported that CML content of plasma of aged hemodialysis patients with non-diabetes is not different from that of aged patients with diabetes²⁷⁾ and that CML content of skin protein in aged subjects is increased in an age-dependent manner.²¹⁾ These results indicate that the CML adduct is a factor promoting neovascularization accumulated in the choroidal explant under both the diabetes mellitus and aging. Especially factors of aging are considered to have a predominant role for the CML-induced choroidal neovascularization rather than factors of diabetes do in the advanced stage of STZ-diabetic rat.

Roles of actions of VEGF, TNF α and PDGF-B for the CML-induced neovascularization were investigated in the period of diabetes. Anti-VEGF and anti-TNF α antibodies inhibited the neovascularization in the early diabetic stage in a time-dependent manner without changing onset time of sprouting. However, anti-PDGF-B antibody significantly delayed the onset time of sprouting microvessel (Fig. 7). These results demonstrate that actions of VEGF and TNF α differed from that of PDGF-B in the action of CML in the diabetic stage. PDGF-B may increase in the migration of cells rather than the proliferation of cells in the choroidal explant of the

early diabetic stage.¹³⁾ In the advanced diabetic stage, the anti-VEGF and anti-TNF α antibodies (Figs. 5, 8) inhibited the CML-induced neovascularization. The inhibitory capacity of anti-TNF α antibody appeared to be greater than that of anti-VEGF antibody. The results demonstrate that the action of CML is mediated by the synergistic action of TNF α and VEGF released from the choroidal explant during the advanced diabetes, being supported by the evidences that activation of AGE receptors leads to increased release of angiogenic factors including VEGF, PDGF and TNF α , all of which may act concomitantly in the process of diabetic angiopathy.^{28—32)} There are another circumstantial evidences that CML is interacted with receptor for AGE (RAGE), releases VEGF and up regulates receptors for AGE, VEGF and TNF α in the vascular endothelial cells,¹⁹⁾ that VEGF can induce choroidal neovascularization and a breakdown of the blood-retinal barrier leading to macular edema,³³⁾ that TNF α increases production of VEGF and its receptor for secondary messengers to induce its neovascularization activity,^{34,35)} and that TNF α is also suggested to have a role in the pathogenesis of proliferative diabetic retinopathy.^{36,37)} We have previously reported that CML-HSA increases immature choroidal microvessels in the culture system by proliferating CD34⁺ cells which are probably endothelial progenitor cells.¹³⁾ There are additional evidences that VEGF differentiates CD34⁺, VE-cadherin⁺ multipotential adult progenitor cell into CD34⁺, VE-cadherin⁺ cell *in vitro*³⁸⁾ and that VEGF increases to initiate the process of vascularization by stimulating chemoattraction and proliferation of CD34⁺ cells such as angioblast and endothelial cell although VEGF alone is not sufficient to direct blood vessel organization and/or maturation.^{39—41)} These evidences suggest that VEGF and TNF α play a role for CML-facilitated proliferation of CD34⁺ cells during the course of diabetes.

Pericytes provide vascular stability and control endothelial proliferation. PDGF-B is involved in pericyte recruitment to a variety of vascular bed.⁴²⁾ The present results demonstrate that the released PDGF-B hastens the onset time of sprouting choroidal microvessels in the early diabetic stage in culture. The number of microvessels in the early diabetic stage was greater than that in the advanced stage on the third day in culture (Fig. 4), indicating that the choroidal microvessel in the early diabetic stage exhibited the shorter onset time of sprouting than that in the advanced diabetic stage. These results suggest that some factors such as PDGF-B may be released from pericytes by CML and facilitate the onset time of sprouting in the early diabetic stage. In addition, the growth curves of microvessels in cultured explants of the early diabetic and normal young control rats had a plateau whereas the number of microvessels of the advanced diabetic and normal aged control rats was continuously increased during the observation period (Fig. 4). These results suggest that the choroidal explants of young rats with and without diabetes mellitus may contain greater number of pericytes than those of aged rats with and without diabetes mellitus. Factors released from pericytes may accelerate the onset time of sprouting and inhibit growth of microvessels in the early diabetic rat. Tissue-accumulated CML might induce damage and loss of pericytes in the choroids of advanced diabetic rat,⁴³⁾ although further investigations need to prove these modification processes.

There is few clinical and histopathological evidences that choroidal neovascularization is an important feature of the disease process in the eyes of human diabetic subjects.⁴⁴⁾ However, the present study demonstrates that the diabetic rat induces overproduction of choroidal neovascularization in culture depending on the duration of diabetes (Fig. 4). The different activity of choroidal capillary between *in vitro* and *in vivo* conditions may be associated with different experimental conditions such as inflammation process, injury of basement membranes, fibrin gel with ϵ -amino caproic acid and lack of circulating nutrients and components in blood. We have unpublished data that the serum of early diabetic rat increased the choroidal neovascularization but the serum of advanced diabetic rats decreased the neovascularization, suggesting that blood deficient condition such as ischemia *in vivo* may enhance the choroidal neovascularization in the advanced diabetes. The anti-angiogenic action of some inhibitor substances in serum of the advanced diabetic rat might keep self-defense mechanisms of choroidal capillaries in the advanced diabetes *in vivo*. To understand pathogenesis of diabetic retinopathy, the interaction of choroidal capillary with retinal neovascularization in the process of diabetes will be issues important.

In conclusion, CML adducts were accumulated and overproduced the actions of VEGF, TNF α and PDGF-B in the choroidal explant during the period of diabetes. TNF α and VEGF are likely to play the predominant role for the CML-induced cell proliferation in the choroidal neovascularization in culture.

Acknowledgments This work was supported in part by a Grant-in-Aid (No 13672310, 15590074) for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture, Japan (to SK) and the Specific Research Fund of Hokuriku University, Kanazawa, Japan (to SK).

REFERENCES

- Forrester J. V., Knott R. M., "Textbook of Diabetes," Chapter 45, ed. by Williams G., Blackwell Science, New York, 1997, pp. 45.1—45.19.
- Brownlee M., *Diabetes*, **43**, 836—841 (1994).
- Bucala R., Cerami A., *Adv. Pharmacol.*, **23**, 1—34 (1992).
- Hammes H. P., Weiss A., Hess S., Araki N., Horiuchi S., Brownlee M., Preissner K. T., *Lab. Invest.*, **75**, 325—338 (1996).
- Monnier V. M., Cerami A., *Science*, **211**, 491—493 (1981).
- Hammes H. P., Alt A., Niwa T., Clausen J. T., Bretzel R. G., Brownlee M., Schleicher E. D., *Diabetologia*, **42**, 728—736 (1999).
- Murata T., Nagai R., Ishibashi T., Inomuta H., Ikeda K., Horiuchi S., *Diabetologia*, **40**, 764—769 (1997).
- Aiello L. P., Avery R. L., Arrigg P. G., Keyt B. A., Jampel H. D., Shah S. T., Pasquale L. R., Thieme H., Iwamoto M. A., Park J. E., *N. Engl. J. Med.*, **331**, 1480—1487 (1994).
- Amin R. H., Frank R. N., Kennedy A., Elliott D., Puklin J. E., Abrams G. W., *Invest. Ophthalmol. Vis. Sci.*, **38**, 36—47 (1997).
- Lian X.-C., Hagino N., Guo S.-S., Tsutsumi T., Kobayashi S., *Phy-tomed*, **9**, 377—384 (2002).
- Hagino N., Kobayashi S., Tsutsumi T., Horiuchi S., Nagai R., Setalo G., Dettrich E., *Brain Res. Bull.*, **62**, 537—547 (2004).
- Kobayashi S., Fukuta M., Kontani H., Yanagita S., Kimura I., *Jpn. J. Pharmacol.*, **78**, 471—478 (1998).
- Kobayashi S., Shinohara H., Tsuneki H., Nagai R., Horiuchi S., *Biol. Pharm. Bull.*, **27**, 1382—1387 (2004).
- Kimura I., Yanagita S., Kobayashi S., Fukuta M., Okabe M., *International Angiol.*, **19**, 26—34 (2000).
- Kobayashi S., Kimura I., Fukuta M., Kontani H., Inaba K., Niwa M., Mita S., Kimura M., *Biol. Pharm. Bull.*, **22**, 360—365 (1999).
- Kobayashi S., Kimura I., Kimura M., *Immunopharmacol.*, **35**, 171—180 (1996).
- Kobayashi S., Luo B., Okabe M., Kimura I., Kimura M., *Biol. Pharm. Bull.*, **19**, 199—202 (1996).
- Kobayashi S., Inaba K., Kimura I., Kimura M., *Biol. Pharm. Bull.*, **21**, 346—349 (1998).
- Yamagishi S., Yonekura H., Yamamoto Y., Katsuno K., Sato F., Mita I., Ooka H., Satozawa N., Kawakami T., Nomura M., Yamamoto H., *J. Biol. Chem.*, **272**, 8723—8730 (1997).
- Ikeda K., Higashi T., Sano H., Jinnouchi Y., Yoshida M., Araki T., Ueda S., Horiuchi S., *Biochemistry*, **35**, 8075—8083 (1996).
- Dunn J. A., McCance D. R., Thorpe S. R., Lyons T. J., Baynes J. W., *Biochemistry*, **30**, 1205—1210 (1991).
- Smith P. R., Thornalley P. J., *Eur. J. Biochem.*, **210**, 729—739 (1992).
- Simmons D. L., Satterthwaite A. B., Tenen D. G., Seed B., *J. Immunol.*, **148**, 267—271 (1992).
- Kimura I., Tsuneki H., Okabe M., Ogasawara M., *Jpn. J. Pharmacol.*, **74**, 303—311 (1997).
- Nagai R., John T., Miyazaki K., Horiuchi S., *Diabetes*, **49** (Suppl. 1), 144A (2000).
- Tanaka N., Yonekura H., Yamagishi S., Fujimori H., Yamamoto Y., Yamamoto H., *J. Biol. Chem.*, **275**, 25781—25790 (2000).
- Miyata T., Fu M.-X., Kurokawa K., Ypersele D. E., Strihou C., Thorpe S. R., Baynes J. W., *Kidney Int.*, **54**, 1290—1295 (1998).
- Haller H., Drab M., Luft F. C., *Clin. Nephrol.*, **46**, 246—255 (1996).
- Ido Y., Chang K. C., Lejeune W. S., Bjercke R. J., Reiser K. M., Williamson J. R., Tilton R. G., *Microcirculation*, **8**, 251—263 (2001).
- Chiarelli F., Spagnoli A., Basciani F., Tumini S., Mezzetti A., Cipollone F., Cuccurullo F., Morgese G., Verrotti A., *Diabet. Med.*, **17**, 650—656 (2000).
- Murata T., Nakagawa K., Khalil A., Ishibashi T., Inomata H., Sueishi K., *Lab. Invest.*, **74**, 819—825 (1996).
- Mamputu J. C., Renier G., *J. Leukoc. Biol.*, **75**, 1062—1069 (2004).
- Vinore S. A., Derevanik N. L., Vinore M. A., Okamoto N., Campochiaro P. A., *Adv. Exp. Med. Biol.*, **476**, 129—138 (2000).
- Bagchi D., Sen C. K., Bagchi M., Atalay M., *Biochemistry*, **69**, 75—80 (2004).
- Yoshida S., Ono M., Shono T., Izumi H., Ishibashi T., Suzuki H., Kuwano M., *Mol. Cell Biol.*, **17**, 4015—4023 (1997).
- Armstrong D., Augustin A. J., Spengler R., Al-Jada A., Nickola T., Grus F., Koch F., *Ophthalmologica*, **212**, 410—414 (1998).
- Spranger J., Meyer-Schwickerath R., Klein M., Schatz H., Pfeiffer A., *Med. Klin.*, **90**, 134—137 (1995).
- Reyes M., Dudek A., Jahagirdar B., Koodie L., Marker P. H., Verfaillie C. M., *J. Clin. Invest.*, **109**, 337—346 (2002).
- Ash J. D., Overbeek P. A., *Dev. Biol.*, **223**, 383—398 (2000).
- Jeong S.-J., Higuchi R., Ono M., Kuwano M., Kim Y.-C., Miyamoto T., *Biol. Pharm. Bull.*, **26**, 1721—1724 (2003).
- Hata-Sugi N., Kawase-Kageyama R., Wakabayashi T., *Biol. Pharm. Bull.*, **25**, 446—451 (2002).
- Hammes H. P., Lin J., Renner O., Shanl M., Lundqvist A., Betsholtz C., Brownlee M., Deutsch U., *Diabetes*, **51**, 3107—3112 (2002).
- Stitt A. W., Li Y. M., Gardiner T. A., Bucala D. B., Vlassara H., *Am. J. Pathol.*, **150**, 523—531 (1997).
- Hidayat A. A., Fine B. S., *Ophthalmology*, **92**, 512—522 (1985).